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(54) **New expression control sequence.**

(57) New expression control sequences useful in the expression of pro- or eukaryotic proteins in prokaryotic organisms are provided comprising a coliphage T5 promoter combined with a DNA sequence which permits the control of promoter activity. Also described are expression vectors containing these expression control sequences and processes using same for the manufacture of pro- or eukaryotic polypeptides.

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New expression control sequence

The present invention relates to new expression control DNA sequences, to expression vectors containing these DNA sequences, to host cells transformed with these expression vectors, to methods for the manufacture of said expression control DNA sequences, expression vectors and transformants, and to methods for producing pro- and eukaryotic proteins by using the new expression control DNA sequences, vectors and transformants.

The level of production of a protein in a host cell is governed by three major factors: the number of copies of its gene within the cell, the efficiency with which those gene copies are transcribed and the efficiency with which the resultant messenger RNA ("mRNA") is translated. Efficiency of transcription and translation is in turn dependent upon the nucleotide sequences which are normally situated ahead of the desired coding sequence or gene. These nucleotide sequences (expression control DNA sequences) define, inter alia, the location at which RNA polymerase interacts (the promoter sequence) to initiate transcription and at which ribosomes bind and interact with the mRNA (the product of transcription) to initiate translation.

Not all such expression control DNA sequences function with equal efficiency. It is thus often of advantage to separate the specific coding sequence or gene for a desired protein from its adjacent nucleotide sequences and to fuse it to other expression control DNA sequences so as to favor higher levels of expression. This having been achieved, the newly-engineered DNA fragment may be inserted into a higher copy number plasmid or a bacteriophage derivative in order to increase the number of gene copies within the cell thereby improving the yield of the desired protein.

Because over-production of even normally non-toxic gene products may be harmful to host cells and lead to decreased stability of particular host-vector systems, an expression control DNA sequence, in addition to improving the efficiency of transcription and translation of cloned genes, should be made controllable so as to allow modulation of expression during bacterial growth. For example, controllable expression control DNA sequences are ones that may be switched off to enable the host cells to propagate without excessive build-up of gene products and later be switched on to promote the expression of large amounts of the desired protein products, which are under the control of those expression control DNA sequences.

Several expression control DNA sequences, which satisfy some of the criteria set forth above, have been employed to express DNA sequences and genes coding for proteins and polypeptides in bacterial hosts. These include, for example, the operator, promoter and ribosome binding and interaction sequences of the lactose operon of E.coli (e.g., K. Itakura et al., "Expression in Escherichia coli of a chemically synthesized gene for the hormone somatostatin", Science, 198, pp. 1056-1063 [1977]; D.V. Goeddel et al., "Expression in Escherichia coli of chemically synthesized genes for human insulin", PNAS USA, 76, pp. 106-110 [1979]), the corresponding sequences of the tryptophan synthetase system of E. coli (J.S. Emtage et al., "Influenza antigenic determinants are expressed from Haemagglutinin genes cloned in Escherichia coli", Nature, 283, pp. 171-174 [1980]; J.A. Martial et al., "Human growth hormone: Complementary DNA cloning and expression in bacteria", Science, 205, pp. 602-607 [1979]) and the major operator and promoter regions of phage λ (H. Bernard et al., "Construction of plasmid cloning vehicles that promote gene expression from the bacteriophage lambda P_L promoter", Gene, 5, pp. 59-76 [1979]; European patent application, publication no. 41767).

The present invention provides a novel and improved expression control DNA sequence comprising a coliphage T5 promoter combined with a DNA sequence which permits the control of promoter activity (regulating sequence).

More precisely the present invention allows to combine a DNA sequence which permits the control of promoter activity with a coliphage T5 promoter while maintaining at the same time the highly efficient promoter function of the coliphage T5 promoter.

In this invention T5 promoters are defined as promoter function mediating DNA sequences occurring in genomes of the coliphage T5 family and functional combinations derived from such sequences.

Naturally occurring T5 promoters are known to be efficient transcriptional initiation signals (A. von Gabain and H. Bujard, "Interaction of E.coli RNA polymerase with promoters of coliphage T5", Molec. gen. Genet., 157, pp. 301-311 [1977]) with the following main properties:

(i) they exhibit a high forward rate constant in the complex formation between the promoter sequence and E.coli RNA polymerase (A. von Gabain and H. Bujard, "Interaction of Escherichia coli RNA polymerase with promoters of several coliphage and plasmid DNAs", PNAS, 76, pp. 189-193 [1979]);

(ii) they initiate RNA synthesis in vivo and in vitro with unusually high rates;

(iii) they contain conserved sequences in 5 regions of the RNA polymerase binding site (H. Bujard et al., "Integration of efficient promoters of the E.coli system into plasmid vectors", in Gene Amplification and Analysis, Vol. 3: Expression of cloned genes in prokaryotic and eukaryotic cells, eds. T.S. Papas, M. Rosenberg, and J.G. Chirikjian; Elsevier New York-Amsterdam-Oxford, pp. 65-87 [1983]) and

(iv) cloned in expression vehicles these promoters are not regulatable.

T5 promoters useful in the present invention are those of the "preearly" "early" and "late" expression class of the phage, especially the sequences described in the disseration of R. Gentz, Universität Heidelberg, 1984: P_{29}, P_{23} (named P_{N25} in the present specification), P_{24}, G_{26}, P_{28a} (named P_{21} in Bujard et al. supra), P_{28b}, G_{22}, G_{1}, G_{11}, G_{19}, G_{10}.

The DNA sequence of some of the preferred T5 promoters mentioned above are indicated in Table I below:

Table 1

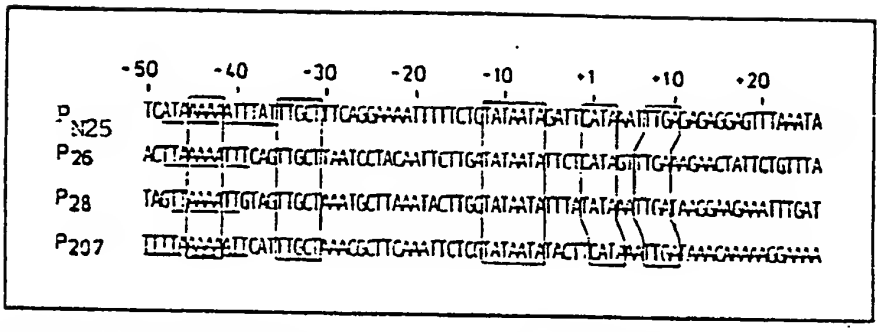

Table 1 shows the nucleotide sequence of these four T5 promoters reacting rapidly with E.coli RNA polymerase. Regions of major homology are boxed. Most striking are the homologies in -10, -33, -43, and +7 regions, as well as the precise spacing (18 bp) between -10 and -33 homologies. The underlined sequences centering around -43 indicate regions where a strong selection against GC base pairs occurs.

DNA sequences which permit the control of the promoter activity are known in the art. They occur in operons (regulatable expression unit) of various types (J.H. Miller and W.S. Reznikoff, "The operon", Cold Spring Harbor Laboratory [1980]). One such type is a negatively controlled expression unit whose essential element is an operator sequence which interacts with a repressor protein. The coding sequence for the repressor protein can be located on a plasmid carrying the operator, or on a separate vector or in the host chromosome.

Preferred DNA sequences which permit the control of the promoter activity, used in this invention, are operator/repressor systems. Especially preferred is the system of the lac operon consisting of the nature lac operator or functional sequences derived therefrom and repressor encoding DNA sequences producing natural or modified repressor molecules (J.H. Miller and W.S. Reznikoff, supra).

The expression control DNA sequences of the present invention can be obtained as follows:

A coliphage T5 promoter sequence can be combined in a manner well known in the art with one or more sequence(s) which permit the control of promoter activity (regulating sequence) to a functional unit whereby the position of the regulating sequences e.g., an operator can vary. In such combinations the regulating sequence can be positioned within or outside the T5 promoter sequence. Thus, a regulating sequence may be integrated within the promoter, may be partially overlapping with or may preceed or follow the promoter at various distances without any overlap. Preferably the regulating sequence overlaps the promoter sequence between position +1 and +20, -13 and -30 and/or -34 and -50 (nomenclature as in table I). Further preferred positions are regions "downstream" of position +20 up to +200. Especially preferred sites are those where the regulating sequence e.g., the lac operator sequence, overlaps the T5 promoter sequence until position +2 in analogy to the lac operon, in E.coli, as shown in Fig. 7.

The expression control DNA sequence of the present invention can be obtained in accordance with methods well-known in DNA chemistry including total chemical synthesis of the respective DNA sequence.

In the preferred embodiment of this invention the lac operator can be negatively regulated by the lac repressor protein which is coded by the lac I-gene. To obtain proper amounts of repressor molecules inside the cell the corresponding gene can be overexpressed by conventional methods, for example by integrating the lac I^q gene (J.H. Miller and W.S. Reznikoff, supra; M.P. Calos, "DNA sequence of a low-level promoter of the lac repressor gene and an 'up' promoter mutation, Nature, 274, pp. 762-765 [1978]) into a vector comprising the above expression control sequence.

The expression sequence of this invention can be introduced in any convenient expression vector of plasmid or phage origin in a manner known per se. Convenient expression vectors of plasmid or phage origin are mentioned e.g., in the laboratory manual "Molecular Cloning" by Maniatis et al., Cold Spring Harbor Laboratory, 1982.

Members of the pDS1-family of plasmids were shown to be suitable for the stable integration of transcriptional signals of exceptional strength (D. Stüber and H. Bujard, "Transcription from efficient promoters can interfere with plasmid replication and diminish expression of plasmid specified genes", The EMBO Journal, 1, pp. 1399-1404 [1982]).

Therefore the preferred vectors used in this invention are those of the pDS1 family. These plasmids are derived from plasmid pBR322 and comprise e.g., pDS1,t_o1^+ and pDS1, t_o 2^+ resulting in plasmid constructions like pDS2/$P_{N25}X_0$,t_o2^+ and pDS3/$P_{N25}X_0$,t_o2^+.

E.coli strains containing plasmids useful for such constructions (E.coli M15 transformed with pDS1,t_o1^+; pDS1,t_o2^+; pDSIX,1) were deposited at Deutsche Sammlung von Mikroorganismen (DSM) in Göttingen on December 11, 1984, the accession nos. being DSM 3135, DSM 3136, DSM 3137 respectively.

The DNA sequences that may be expressed by the expression vectors of this invention may be selected from a large variety of DNA sequences that encode prokaryotic or eukaryotic polypeptides in vivo or in vitro. For example, such sequences may encode enzymes, hormones, polypeptides with immuno-modulatory, anti-viral or anti-cancer properties, antibodies, antigens, vaccines and other useful polypeptides of prokaryotic or eukaryotic origin.

Examples of proteins which can be expressed by using the improved expression control system of the present invention are dihydrofolate reductase, chloramphenicol acetyltransferase, malaria surface antigens, lymphokins like IL-2, interferons α, β and γ, insulin and insulin precursors, growth hormones, tissue plasminogen activator or human renin.

Methods for expressing DNA sequences coding for prokaryotic or eukaryotic proteins using the expression vectors of this invention are well known (Maniatis et al., supra). They include transforming an appropriate host with the expression vector having the desired DNA sequence operatively linked to the expression control sequence of the vector, culturing the host under appropriate conditions of growth and isolating the desired polypeptide from the cul-

ture. Those of skill in the art may select from these known methods those that are most effective for a particular gene expression without departing from the scope of this invention.

The selection of a particular host for use in this invention is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the proteins encoded for by the hybrid plasmid, ease of recovery of the desired protein, expression characteristics, biosafety and costs. Within these general guidelines, examples of useful bacterial hosts are gram-negative and gram-positive bacteria, especially strains of E.coli and B.subtilis. The most preferred host cell of this invention is E.coli M15 (described as DZ291 in M.R. Villarejo et al., "β-Galactosidase from termination and deletion mutant strains", J. Bacteriol., 120, pp. 466-474 [1974]). However other E.coli strains such as E.coli 294 (ATCC No. 31446) and E.coli RR1 (ATCC No. 31343) can also be used.

The present invention will be better understood on the basis of the following examples when considered in connection with the following figures:

The abbreviations and symbols used are: B, E, H, P, X and Xb which indicate sites for restriction endonucleases BamI, EcoRI, HindIII, PstI, XhoI and XbaI, respectively. represents promoters; represents ribosomal binding sites; represents terminator t_o ; represents lac operator (O) or parts of it; represents promoter P_{N25} (P_{N25}); represents region required for DNA-replication (repl.); represents coding region either for dihydrofolat reductase (dhfr), chloramphenicol acetyltransferase (cat), β-lactamase (bla) and lac repressor (lacI) or for parts of the coding region for chloramphenicol acetyltransferase (cat'); represents parts of the coding region for β-galactosidase (lac Z').

Figure 1. Part a) is a schematic drawing of the plasmid pDS1,t_o1^+. The nucleotide sequence of the EcoRI/XbaI-fragment containing the dhfr-gene, terminator t_o and the cat-gene is displayed in part b). Here the restriction endonuclease sites indicated in part a) are overlined. In addition the pBR322 entity of pDS1,t_o1^+ is schematically shown, where the given numbers refer to the nucleotide sequence of pBR322 (J.G. Sutcliffe, "Complete nucleotide sequence of the Escherichia coli plasmid pBR322", Cold Spring Harbor Symp. Quant. Biol., 43, pp. 77-90 [1979].

Figure 2 is a schematic drawing of the plasmid pDS1/P_{N25},t_o1^+. The nucleotide sequence of the XhoI-fragment carrying promoter P_{N25} is displayed. The site of initiation and the direction of transcription are indicated by the arrow.

Figure 3 is a schematic drawing of the plasmid pEX07 lac op 2. The nucleotide sequence of the EcoRI/HindIII-fragment carrying the lac operator is displayed.

Figure 4. Part a) is a schematic drawing of the plasmid pDS1,t_o2^+. The nucleotide sequence of the XhoI/XbaI-fragment containing the dhfr-gene, the cat-gene and terminator t_o is displayed in part b). Here the restriction endonuclease sites indicated in part a) are overlined. In addition the pBR322 entity of pDS1,t_o2^+ is schematically shown, where the given numbers refer to the nucleotide sequence of pBR322 (J.G. Sutcliffe, supra).

Figure 5. Part a) is a schematic drawing of the plasmid pDSIX,1. The nucleotide sequence of the EcoRI/XbaI-fragment containing the lac I-gene, parts of the lac Z-gene (lac Z') and the cat-gene is displayed in parts b) and c). Here the restriction endonuclease sites indicated in part a) are overlined. In addition the pBR322 entity of pDSIX,1 is schematically shown, where the given numbers refer to the nucleotide sequence of pBR322 (J.G. Sutcliffe, supra).

Figure 6a,b,c is a schematic outline of the construction of the promoter/operator fusion $P_{N25}X_0$ embedded in the plasmid pDS2/$P_{N25}X_0$,t_o2^+.

Figure 7 displayes the nucleotide sequence of the XhoI/EcoRI fragment containing the promoter/operator fusion $P_{N25}X_0$. The site of initiation and the direction of transcription are indicated by the arrow.

Figure 8a,b,c is a schematic outline of the construction of plasmids pDSXII,3, pDS3/$P_{N25}X_0$,t_o 2^+ and pDSXIII,1.

Figure 9 is an electropherogram monitoring protein synthesis in the absence or presence of IPTG in E.coli M15 harboring plasmids pDSXII,3 (lane 1), pDS2/P_{N25} X_0,t_o2^+ (lanes 2 and 3) or pDS3/$P_{N25}X_0$,t_o2^+ (lanes 4-7).

The construction of the promotor/operator fusion PN $_{N25}X_0$ of this invention is described more in detail in the following examples:

Example 1

Description of plasmids used for the construction of promoter P_{N25} X_0

A. Principles

pDS1, t_o1^+ (figure 1) and pDS1, t_o2^+ (figure 4) were chosen as vectors not only for the integration of promoter P_{N25} and the construction of the fusion between promoter $P_{N25}$and the lac operator but also for the demonstration of the function of the resulting promoter/operator fusion $P_{N25}X_0$.

Although there are several well defined sources for promoter P_{N25}, plasmid pDS1/P_{N25},t_o1^+ (figure 2) carrying this element on an EcoRI-fragment was chosen as source for promoter P_{N25} whereas pEX07 lac op 2 (figure 3; H. Weiher, "Untersuchungen zur Struktur und Funktion von E.coli Promotoren: Variation des Lac Promoters durch gezielte Neukonstruktion von Teilsequenzen und Mutagenese", Ph.D. thesis, University of Heidelberg, FRG [1980]) comprising 21 basepairs of the lac operator was chosen to provide the operator entity in the promoter/operator fusion. The lac repressor is encoded by the lac I gene. Since a promoter is efficiently repressed by binding of repressor to the operator only if sufficient amounts of repressor molecules are present, the lac I^q allel was used with a mutant promoter resulting in an increased transcription of the gene to provide enough repressor molecules. As source of this mutant lac I gene plasmid pIX,1 was chosen (figure 5).

B. Plasmid pDS1,t_o1^+

The part of pDS1,t_o1^+ (figure 1; D. Stüber and H. Bujard, supra) between the sites for restriction endonucleases XbaI and EcoRI containing the region required for DNA replication and maintenance of the plasmid in the cell and the entire gene for β-lactamase conferring resistance to ampicillin is pBR322 derived (F. Bolivar et al., "Construction and characterization of new cloning vehicles II. A multi-purpose cloning system", Gene, 2, pp. 95-113 [1977]; J.G. Sutcliffe, supra). The remaining part of the plasmid carries sites for restriction endonucleases XhoI, EcoRI and BamHI followed by the coding region for dihydrofolate reductase of mouse AT-3000 cell line (A.C.Y. Chang et al., "Phenotypic expression in E. coli of a DNA sequence coding for mouse dihydrofolate reductase", Nature, 275, pp. 617-624 [1978]; J.N. Masters and G. Attardi, "The nucleotide sequence of the cDNA coding for the human dihydrofolic acid reductase", Gene, 21, pp. 59-63 [1983]), by the terminator t_o of phage lambda (E. Schwarz et al., "Nucleotide sequence of cro, cII and part of the O gene in phage λ DNA", Nature, 272, pp. 410-414 [1978]; D. Stüber and H. Bujard, supra) and by the promoter-free gene for chloramphenicol acetyltransferase (R. Marcoli et al., "The DNA sequence of an IS1-flanked transposon coding for resistance to chloramphenicol and fusidic acid", FEBS Letters, 110, pp. 11-14 [1980]).

C. Plasmid pDS1/P_{N25},t_o1^+

Plasmid pDS1/P_{N25},t_o1^+ (figure 2) corresponds to pDS1,t_o1^+ (figure 1) but contains on an XhoI-fragment promoter P_{N25} of E.coli phage T5 (A. von Gabain and H. Bujard, supra; D. Stüber et al., "Electron microscopic analysis of in vitro transcriptional complexes: Mapping of promoters of the coliphage T5 genome", Molec. gen. Gen., 166, pp. 141-149 [1978]; H. Bujard et al., supra).

D. Plasmid pEX07 lac op 2

Plasmid pEX07 lac op 2 (figure 3; H. Weiher, supra) is a derivative of pBR322 (F. Bolivar et al., supra; J.G. Sutcliffe, supra) where the EcoRI/HindIII-fragment of pBR322 is replaced by an EcoRI/HindIII-fragment containing 21 basepairs of the lac operator sequence (J.H. Miller and W.S. Reznikoff, supra).

E. Plasmid pDS1,t_o2^+

The part of pDS1,t_o2^+ (figure 4; D. Stüber and H. Bujard, supra) between the sites for restriction endonucleases XbaI and XhoI containing the region required for DNA replication and maintenance of the plasmid in the cell and the entire gene for β-lactamase confering resistance to ampicillin is pBR322 derived (F. Bolivar et al., supra; J.G. Sutcliffe, supra). The remaining part of the plasmid carries sites for restriction endonucleases EcoRI and BamHI followed by the coding region for dihydrofolate reductase of mouse AT-3000 cell line (A.C.Y. Chang et al., supra; J.N. Masters and G. Attardi, supra), by the promoter-free gene for chloramphenicol acetyltransferase (R. Marcoli et al., supra) and by the terminator t_o of phage lambda (E. Schwarz et al., supra).

F. Plasmid pDSIX,1

Plasmid pDSIX,1 (figure 5) carries the identical part of pBR322 as plasmid pDS1,t_o1^+ (figure 1). In addition it contains the entire gene for the lac repressor (P.J. Farabaugh, "Sequence of the lacI gene", Nature, 274, pp. 765-769 [1978]) with the promoter mutation I^q (M.P. Calos, supra) followed by sites for restriction endonucleases EcoRI and XhoI, by the regulatory and part of the coding region of the gene for β-galactosidase (A. Kalnins et al., "Sequence of the lac Z gene of Escherichia coli", The EMBO J., 2, pp. 593-597 [1983]; R. Gentz et al., "Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal", PNAS, 78, pp. 4936-4940 [1981]) and by the promoter-free gene for chloramphenicol acetyltransferase (R. Marcoli et al., supra).

Example 2

Construction of the plasmid pDs2/$P_{N25}x_{o}$,t_o2^+ carrying the promoter/operator fusion P_{N25} x_{o}

The promoter/operator fusion $P_{N25}x_{o}$ was prepared and embedded in pDS1,t_o 2^+ to give pDS2/$P_{N25}x_{o}$,t_o 2^+ in a sequence of steps. These are depicted in figure 6 and more fully described below.

A. Preparation of the promoter entity of the promoter/operator fusion

a) Isolation of the EcoRI-fragment containing P_{N25}

To isolate the EcoRI-fragment carrying promoter P_{N25}, 32.5 µg of plasmid pDS1/P_{N25},t_o1^+ were digested with EcoRI (figure 6a) and after extraction with phenol followed by treatment with ether and subsequent precipitation with ethanol the DNA fragments were separated by electrophoresis in a 6% polyacrylamid gel. Following visualization of the DNA by staining with ethidium bromide, the fragment carrying P_{N25} was cut out of the gel and eluted by shaking the mashed gel slice for 10 hours in a buffer containing 2.5 mM Tris/HCl (pH 7.6) and 0.25 mM EDTA. After removal of the gel pieces by centrifugation, the resulting supernatant was extracted with phenol, treated with ether and the DNA fragment collected by precipitation with ethanol. 1.3 µg of the promoter-fragment were isolated in this way.

b) Limited digestion of the EcoRI-fragment with HinfI

1 µg of the EcoRI-fragment was incubated in a volume of 120 µl in a buffer containing 100 mM sodium chloride with 21 units HinfI for 9 minutes at 37°C. The enzyme was inactivated by incubation of the mixture for 7 minutes at 65°C and withdrawn by three extractions with phenol. After removal of the residual phenol by extraction with ether the DNA was precipitated with ethanol and stored in a buffer containing 5 mM Tris/HCl (pH 7.6), 0.5 mM EDTA and 100 mM sodium chloride. Analysis of the DNA by electrophoresis in a 6% polyacrylamid gel revealed that about 60% of the EcoRI-fragment molecules had been cleaved by HinfI.

c) Extending the recessed 3'-ends with DNA polymerase I

0.17 µg of the HinfI digested DNA was incubated in a

volume of 25 µl in a buffer consisting of 100 mM potassium phosphate (pH 7.0), 100 mM potassium chloride, 7 mM magnesium chloride, 25 µM each of the four deoxynucleoside-triphosphates with 5 units of DNA polymerase I for 40 minutes at 20°C. After removal of the enzyme by extraction with phenol, the solution was chromatographed through Sephadex G75 equilibrated in a buffer of pH 7.7 consisting of 2.5 mM Tris, 0.25 mM EDTA and 50 mM potassium acetate. DNA containing fractions were combined before the DNA was precipitated with ethanol and stored in ligation buffer supplemented with 50 mM sodium chloride. The resulting solution with 0.08 µg DNA contained fragment 1 (figure 6a).

B. Preparation of the operator entity of the promoter/operator fusion

a) Limited digestion of pEX07 lac op 2

14.5 µg of plasmid pEX07 lac op 2 (figure 6b) were incubated in a volume of 250 µl with 1.5 units EcoRI for 50 minutes at 37°C. The enzyme was inactivated by incubation for 7 minutes at 65°C and withdrawn by extraction with phenol. After removal of residual phenol with ether the DNA was precipitated with ethanol and stored in a buffer consisting of 2.5 mM Tris/HCl (pH 7.6) and 0.25 mM EDTA. Analysis of the DNA by electrophoresis in a 6% polyacrylamid gel revealed that about 50% of the plasmid molecules had been linearized.

b) Removal of the 5'-protruding ends with S1 nuclease To remove the extended 5'-ends, the EcoRI digested DNA was treated with the single-strand specific nuclease S1 (figure 6b). In three separate experiments 1.7 µg DNA were incubated in a volume of 50 µl in a buffer containing 280 mM sodium chloride, 30 mM sodium acetate (pH 4.4), 4.5 mM zinc acetate and 20 µg /ml single-stranded DNA of phage fd with 160 units, 800 units and 4000 units S1 nuclease (Boehringer, Mannheim, FRG) for 30 minutes at 20°C. The reactions were stopped by adding ammonium acetate and EDTA to final concentrations of 0.5 M and 10 mM, respectively, and incubation for 7 minutes at 65°C. After removal of the protein by extraction with phenol followed by treatment with ether, the three mixtures were combined and the resulting solution chromatographed through Sephadex G75 equilibrated in a buffer of pH 7.0 consisting of 2.5 mM Tris/HCl, 0.25 mM EDTA and 50 mM potassium acetate. DNA containing fractions were combined before the DNA was precipitated with ethanol and stored in ligation buffer. The resulting solution with 4.3 µg DNA contained fragment 2 (figure 6b).

C. Preparation of plasmid pDS1,t_o2^+ accepting the promoter/operator fusion

pDS1,t_o2^+ (figure 4) was chosen as vector to accept the promoter/operator fusion. Therefore, 5.1 µg DNA of this plasmid were digested to completion with restriction endonucleases HindIII and XhoI (figure 6c). After inactivation of the enzymes by incubation for 7 minutes at 65°C and removal of the protein by extraction with phenol followed by treatment with ether, the DNA was precipitated with ethanol and stored in a buffer consisting of 2.5 mM Tris/HCl (pH 7.6) and 0.25 mM EDTA.

D. Construction of pDS2/P_{N25X_0},t_o2^+

To construct pDS2/P_{N25X_0},t_o2^+ the promoter entity (fragment 1) and the operator entity (fragment 2) of P_{N25X_0} were ligated together and the resulting fusion subsequently embedded in pDS1,t_o2^+ (figure 6c). For the fusion of promoter and operator 0.05 µg of the DNA mixture containing fragment 1 and 3.5 µg of the DNA mixture containing fragment 2 were incubated in ligation buffer supplemented with 25 mM sodium chloride with 5 units T4-DNA ligase for 7 hours at 15°C. After inactivation of the enzyme by incubation for 7 minutes at 65°C, the ligation mixture was incubated in a volume of 100 µl with 20 units HindIII and 15 units XhoI for 1 hour at 37°C to obtain the promoter/operator fusion as an XhoI/HindIII fragment. After inactivation of the restriction endonucleases at 65°C and their removal by extraction with phenol followed by treatment with ether, the DNA was precipitated with ethanol and redissolved in ligation buffer supplemented with 25 mM potassium chloride. 0.25 µg plasmid pDS1,t_o2^+ digested with HindIII as well as XhoI and 1.3 units T4-DNA ligase were added and the mixture incubated for 4 hours at 15°C before the enzyme was inactivated by incubation for 7 minutes at 65°C.

E.coli C600 (CaCl, competent) was transformed with half of the above prepared ligation mixture under appropriate conditions. Transformants were selected at 37°C on Minimal Agar Plates based on M9-medium (J.H. Miller, "Experiments In Molecular Genetics", Cold Spring Harbor Laboratory, pp. 431-432 [1972]) supplemented with 0.2% glucose, 0.5% casein hydrolysate, 10 mg/liter vitamin B1, 40 mg/liter X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 10-200 µg/ml chloramphenicol.

Only transformants containing plasmid pDS1,t_o2^+ with a promoter integrated in front of the promoter-free gene for chloramphenicol acetyltransferase were expected to grow on plates with concentrations of chloramphenicol of more than 10 µg/ml. Furthermore, transformants containing pDS1,t_o2^+ with P_{N25X_0} integrated in front of this indicator gene were expected to become blue on these plates, due to the induction of the host lac system by binding of the lac repressor molecules to the operator sequence present in the plasmids and subsequent cleavage of the colourless X-gal to a blue derivative. Therefore, 18 blue transformants from plates with 50 µg/ml or higher concentrations of chloramphenicol were selected and cultures grown at 37°C in LB-medium containing 100 µg/ml ampicillin. DNA from these cultures was isolated using standard procedures and analyzed for its size and the presence of sites for restriction endonucleases EcoRI, HindIII, HinfI and XhoI. One plasmid displaying the expected patterns after electrophoresis of restriction fragments in 6% polyacrylamid gels was designated pDS2/P_{N25X_0},t_o2^+ (figure 6c). Sequence analysis of the HindIII-XhoI fragment of this plasmid confirmed the expected sequence for promoter P_{N25X_0} as depicted in figure 7.

Example 3

Construction of plasmids pDSXII,3, pDS3,$P_{N25}x_0$,to2$^+$ and pDSXIII,1 containing promoter $P_{N25}x_0$

In addition to pDS2/$P_{N25}x_0$,t_o2^+ (lacking repressor gene) three more plasmids containing the promoter/operator fusion $P_{N25}x_0$, namely pDSXII,3 (intermediate for the manufacture of pDS XIII,1), pDS3/$P_{N25}x_0$,t_o2^+ (as an example of plasmids according to the invention) and pDSXIII,1 (with a partly defeted cat-gene lacking RBS; as a negative control for comparative purposes), were designed to demonstrate the function of this element. The construction of these plasmids is depicted in figure 8 and more fully described below.

A. Construction of plasmid pDSXII,3

Plasmid pDSXII,3 was derived from pDS2/$P_{N25}x_0$,t_o2^+ by eliminating the EcoRI-fragment of this plasmid containing the single site for restriction endonucleases HindIII and part of the gene for chloramphenicol acetyltransferase (figure 8a). For that purpose 0.45 µg of pDS2/P_{N25} x_0,t_o2^+ were digested to completion with restriction endonuclease EcoRI. After removal of the enzyme by extraction with phenol, the solution was chromatographed through Sephadex G75 equilibrated in a buffer of pH 7.6 consisting of 2.5 mM Tris/HCl and 0.25 mM EDTA. DNA containing fractions were combined and the resulting solution was concentrated by lyophilization. 0.13 µg of the plasmid digested with EcoRI were incubated in a volume of 100 µl in ligation buffer with 1.3 units T4-DNA ligase for 5 hours at 15°C before the enzyme was inactivated by incubation for 7 minutes at 65°C.

E.coli M15 ($CaCl_2$ competent) was transformed with 0.07 µg of the ligated DNA under appropriate conditions. Transformants were selected at 37°C on Minimal-Agar plates based on M9-medium (J.H. Miller, supra) supplemented with 0.2% glucose, 0.5% casein hydrolysate, 10 mg/liter vitamin B1, 40 mg/liter X-gal and 1 mM IPTG (Isopropylthiogalactoside) and containing either 100 µg/ml ampicillin or 50 µg/ml chloramphenicol.

Since loss of the EcoRI-fragment comprising part of the gene for chloramphenicol acetyltransferase results in loss of resistance to chloramphenicol, plates with ampicillin were found to have about 40 times more transformants than plates with ampicillin. 4 transformants resistant to ampicillin were selected and cultures grown in LB-medium containg 100 µg/ml ampicillin. DNA from these cultures was isolated using standard procedures and analyzed for its size and presence of sites for restriction endonucleases EcoRI, HindIII, HinfI, PstI and XhoI. One plasmid displaying the expected patterns after electrophoresis in 6% polyacrylamid-gels was designated pDSXII,3 (figure 8a).

B. Construction of plasmids pDS3/$P_{N25}x_0$,t_o2^+ and pDSXIII,1

Plasmids pDS3/$P_{N25}x_0$,t_o2^+ and pDSXIII,1 were derived by combining appropriate fragments of plasmids pDSIX,1 and pDS2/$P_{N25}x_0$,t_o2^+ (figure 8b) or pDSXII,3 (figure 8c), respectively. For that purpose, 1.25 µg of these three plasmids were digested in three separate reactions to completion with restriction endonucleases PstI and XhoI. After removal of these enzymes by extraction with phenol, the three solutions were chromatographed through Sephadex G75 equilibrated in a buffer of pH 7.6 consisting of 2.5 mM Tris/HCl and 0.25 mM EDTA. DNA containing fragments of each experiment were combined and the resulting solutions concentrated by lyophilization.

In two separate reactions 0.13 µg digested DNA of each of the plasmids pDSIX,1 and pDS2/$P_{N25}x_0$,t_o2^+ or pDSIX,1 and pDSXII,3 were incubated in a volume of 60 µl in ligation buffer with 1.3 units T4-DNA ligase for 10 hours at 15°C before the enzyme was inactivated by incubation for 7 minutes at 65°C. Again in two separate reactions E.coli M15 (CaCl, competent) was transformed with 0.12 µg of the ligated DNA under appropriate conditions. Transformants were selected at 37°C on Minimal-Agar plates based on M9-medium (J.H. Miller, supra) supplemented with 0.2% glucose, 0.5% casein hydrolysate, 10 mg/liter vitamin B1, 40 mg/liter X-gal and 1 mM IPTG and containing 100 µg/ml ampicillin. Six white transformants of each transformation were selected and cultures grown in LB-medium containing 100 µg/ml ampicillin. DNA of these cultures was isolated using standard procedures and analyzed for its size and presence of sites for restriction endonucleases EcoRI, HindIII, HinfI, PstI and XhoI. Two plasmids derived from pDS2/$P_{N25}x_0$,t_o2^+ and pDSXII,3 displaying the expected patterns after electrophoresis in 6% polyacrylamid gels were designated pDS3/$P_{N25}x_0$,t_o2^+ (figure 8b) and pDSXIII,1 (figure 8c), respectively.

Example 4

Expression of chloramphenicol acetyltransferase in plamids containing promoter/operator fusion P_{N25} x_0

To demonstrate the usefulness of promoter/operator fusion $P_{N25}x_0$, the synthesis of chloramphenicol acetyltransferase in cells harboring plasmids containing $P_{N25}x_0$was monitored. E.coli M15 cells transformed with either pDS2/$P_{N25}x_0$,t_o2^+, pDSXIII,1 or pDS3/$P_{N25}x_0$, t_o2^+ were grown in the presence or absence of 1 mM IPTG in LB-medium containing 100 µg/ml ampicillin at 37°C to a density of $1.7 \cdot 10^9$ cells/ml. $1.5 \cdot 10^8$ cells were collected by centrifugation and resuspended in sample buffer containing 1% SDS, 1% β-mercaptoethanol, 10% glycerol and 62.5 mM Tris/HCl (pH 6.8). Samples were boiled for 5 minutes, chilled on ice, centrifuged at 12 000 xg for 30 seconds and electrophoresed in a SDS-containing polyacrylamide gel (12.5% acrylamide) according to the procedure of U. Laemmli, "Cleavage of structural proteins during the assembly of the head of Bacteriophage T4", Nature, 227, pp. 680-682 [1970]. After staining of the proteins with Coomassie brillant Blue R-250 the unbound dye was removed from the gel. A fotograph of this gel is shown in figure 9.

Comparing now lanes 1, 2 and 3 of figure 9 it becomes obvious that as expected pDSXIII,1 (lane 1) encodes lac repressor (R) but not chloramphenicol acetyltransferase (CAT) whereas in cells transformed with pDS2/$P_{N25}x_0$,t_o2^+ (lanes 2 and 3) the repressor is absent and chloramphenicol acetyltransferase is produced in large amounts independent of the presence of IPTG in the medium. Although pDS3/$P_{N25}x_0$,t_o2^+ differs from pDS2/$P_{N25}x_0$,t_o2^+ only in comprising the gene for the lac repressor (figure 8b), in cells transformed with pDS3/$P_{N25}x_0$,t_o2^+ only unvisible amounts of chloramphenicol acetyltransferase are produced in the absence of IPTG (compare lanes 4 and 5 with lanes 1, 2 and 3). However, in cells harboring pDS3/$P_{N25}x_0$,t_o2^+

and grown in the presence of IPTG essentially the same amount of chloramphenicol acetyltransferase is produced as in cells containing pDS2/P $_{N25X_0,t_o2^+}$ (compare lanes 6 and 7 with lanes 2 and 3).

The results described above show, that promoter P_{N25X_0} is repressed in the presence of lac repressor and is induced essentially to full activity by addition of the inducer IPTG.

Claims

1. An expression control DNA sequence comprising a coliphage T5 promoter combined with a DNA sequence which permits the control of promoter activity.

2. An expression control DNA sequence according to claim 1, wherein said coliphage T5 promoter is the P_{N25} promoter.

3. An expression control DNA sequence according to claim 1 or 2 wherein the DNA sequence which permits the control of promoter activity is an operator.

4. An expression control DNA sequence according to claim 1, 2 or 3 wherein said operator is the lac operator or a functional part thereof.

5. An expression control DNA sequence according to claim 4, wherein the lac operator or a functional part thereof overlaps the T5 promoter sequence.

6. An expression control DNA sequence according to any one of claims 1 to 5 comprising the functional part of the nucleotide sequence of fig. 7.

7. An expression vector comprising an expression control DNA sequence according to claims 1 to 6.

8. An expression vector according to claim 7 which is a plasmid capable of replication in gram-negative and/or gram-positive bacteria.

9. An expression vector according to claim 8 which is capable of replication in an E.coli strain.

10. An expression vector according to claim 8 which is capable of replication in a B.subtilis strain.

11. An expression vector according to claim 8 or 9 which is a member of the pDS 1 plasmid family.

12. An expression vector of claim 11 which is pDS2/P $_{N25X_0,t_o2^+}$.

13. An expression vector of claim 11 which is pDS3/P_{N25X_0} ,t_o2^+.

14. A transformant carrying an expression vector claimed in any one of claims 7 to 13.

15. A transformant according to claim 14 which is an E.coli strain.

16. A transformant according to claim 15 which is an E.coli M15 strain.

17. A transformant according to claim 14 which is a B.subtilis strain.

18. A process for the manufacture of a pro- or eukaryotic polypeptide which process comprises transforming a host with an expression vector containing the DNA sequence coding for said polypeptide operatively linked to an expression control DNA sequence as claimed in any one of claims 1 to 6, culturing the transformant under appropriate conditions of growth and isolating the desired polypeptide from the culture.

19. A process according to claim 18 wherein the vector is a plasmid capable of replication in gram-negative and/or gram-positive bacteria.

20. A process according to claim 19 wherein the plasmid is capable of replication in an E.coli strain.

21. A process according to claim 19 wherein the plasmid is capable of replication in a B.subtilis strain.

22. A process according to claim 19 or 20 wherein the plasmid is a member of the pDS1 family.

23. A process according to claim 22 wherein the plasmid is pDS2/P_{N25X_0,t_o2^+}.

24. A process according to claim 22 wherein the plasmid is pDS3/P_{N25X_0,t_o2^+}.

25. A process according to claims 18 to 24 wherein the polypeptide is chloramphenicol acetyltransferase.

26. The use of an expression control DNA sequence as claimed in any one of claims 1 to 6 for expressing a pro- or eukaryotic polypeptide.

Claims for contracting State: AT

1. A process for the preparation of an expression control DNA sequence comprising a coliphage T5 promoter combined with a DNA sequence which permits the control of promoter activity, which process comprises combining a coliphage T5 promoter in a manner well known in the art with a DNA sequence which permits the control of promoter activity to a functional unit.

2. A process according to claim 1, wherein said coliphage T5 promoter is the P_{N25} promoter.

3. A process according to claim 1 or 2 wherein the DNA sequence which permits the control of promoter activity is an operator.

4. A process according to claim 1, 2 or 3 wherein said operator is the lac operator or a functional part thereof.

5. A process according to claim 4, thereby creating an expression control DNA sequence characterized in that the lac operator or a functional part thereof overlaps the T5 promoter sequence.

6. A process according to any one of claims 1 to 5, thereby creating an expression control DNA sequence comprising the functional part of the nucleotid sequence of fig. 7.

7. A process for the preparation of an expression vector comprising a DNA portion corresponding to an expression control DNA sequence obtained according to any one of claims 1-6, which process comprises providing said DNA portion and introducing said DNA protion into a convenient expression vector.

8. A process acccording to claim 7, wherein said convenient expression vector is a plasmid capable of replication in gram-negative and/or gram-positive bacteria.

9. A process according to claim 8, wherein said plasmid is capable of replication in an E. coli strain.

10. A process according to claim 8, wherein said plasmid is capable of replication in a B. subtilis strain.

11. A process according to claim 8 or 9, wherein said plasmid is a member of the pDS 1 family.

12. The process of claim 11, wherein said plasmid is pDS2/$P_{N25}x_{o}.t_{o}2^{+}$.

13. The process of claim 11, wherein said plasmid is pDS3/$P_{N25}x_{o}.t_{o}2^{+}$.

14. A process for the preparation of a transformant carrying an expression vector obtained according to any one of claims 7 to 13 which process comprises transforming a vector with said expression vector by methods known in the art.

15. A process according to claim 14, wherein said vector is an E. coli strain.

16. A process according to claim 15, wherein said E. coli strain is an E. coli M15 strain.

17. A process according to claim 14, wherein said vector is a B. subtilis strain.

18. A process for the manufacture of a pro- or eukaryotic polypeptide which process comprises transforming a host with an expression vector containing the DNA sequence coding for said polypeptide operatively linked to an expression control DNA sequence obtained according to any one of claims 1 to 6, culturing the transformant under appropriate conditions of growth and isolating the derived polypeptide from the culture.

19. A process according to claim 18 wherein the vector is a plasmid capable of replication in gram-negative and/or gram-positive bacteria.

20. A process according to claim 19 wherein the plasmid is capable of replication in an E.coli strain.

21. A process according to claim 19 wherein the plasmid is capable of replication in a B.subtilis strain.

22. A process according to claim 19 or 20 wherein the plasmid is a member of the pDS1 family.

23. A process according to claim 22 wherein the plasmid is pDS2/$P_{N25}x_{o}.t_{o}2^{+}$.

24. A process according to claim 22 wherein the plasmid is pDS3/$P_{N25}x_{o}.t_{o}2^{+}$.

25. A process according to claims 18 to 24 wherein the polypeptide is chloramphenicol acetyltransferase.

Fig. 1a

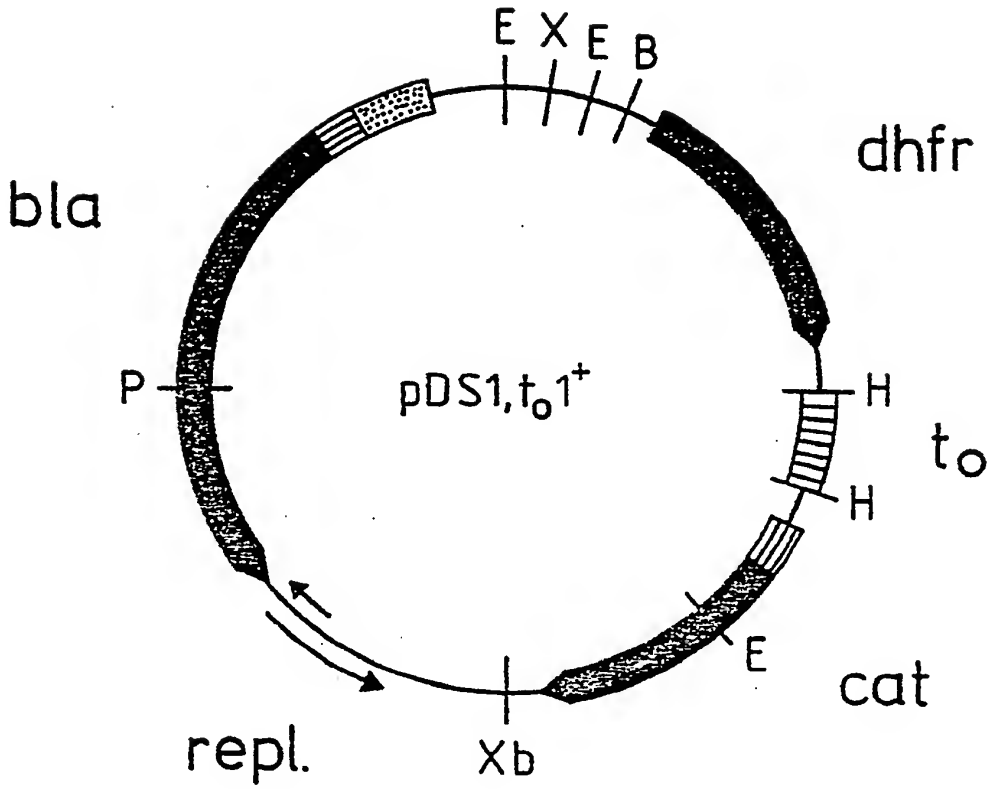

Fig1b

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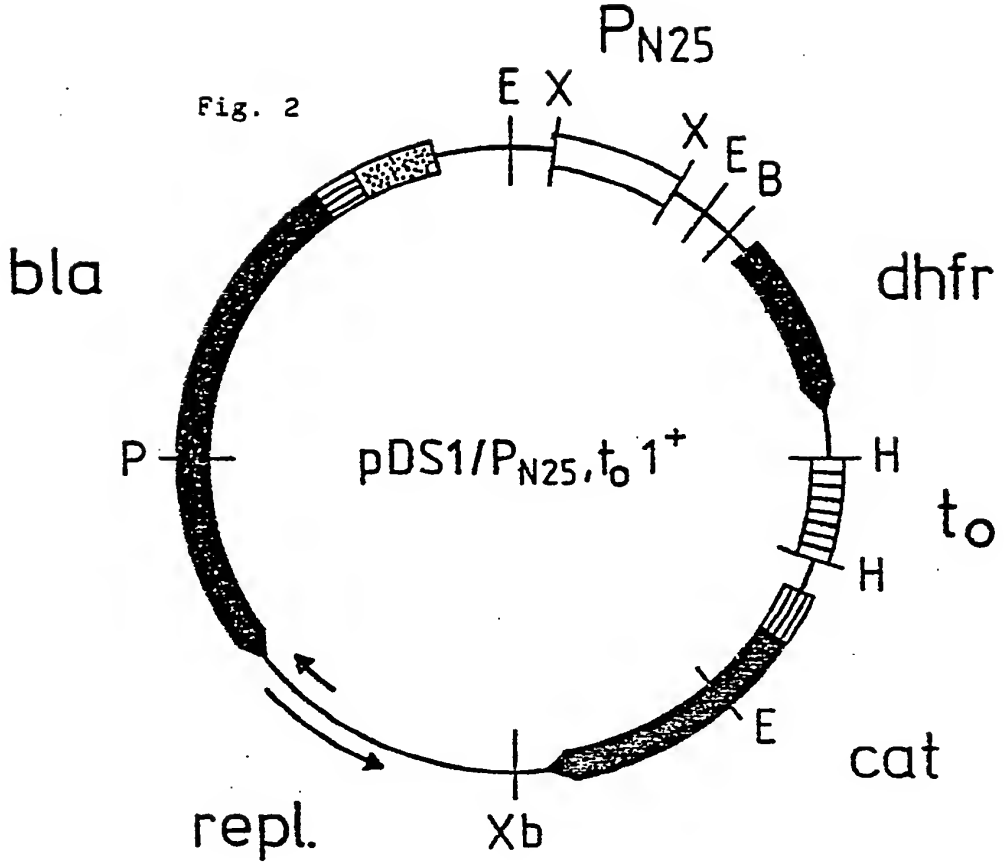
Fig. 2

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                      HinfI
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                  HinfI
150 AAATTTTTCT GTATAATAGA TTCATAAATT TGAGAGAGGA GTTTAAATAT

                                                  XhoI
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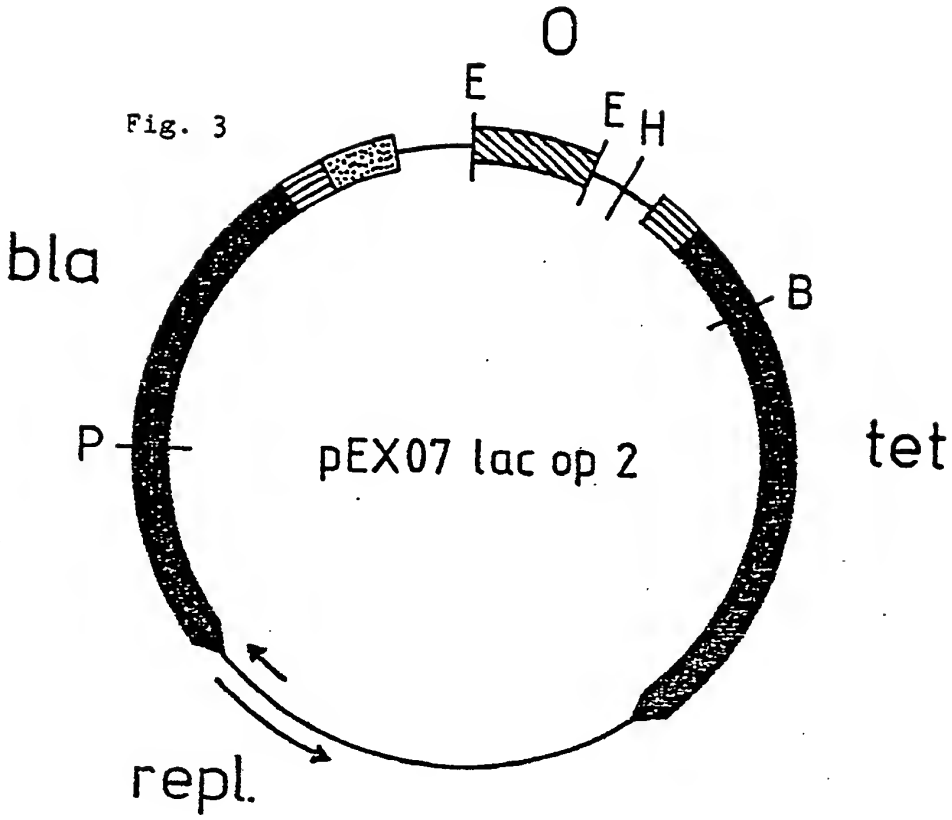
Fig. 3



operator

EcoRI — EcoRI HindIII

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Fig. 4a

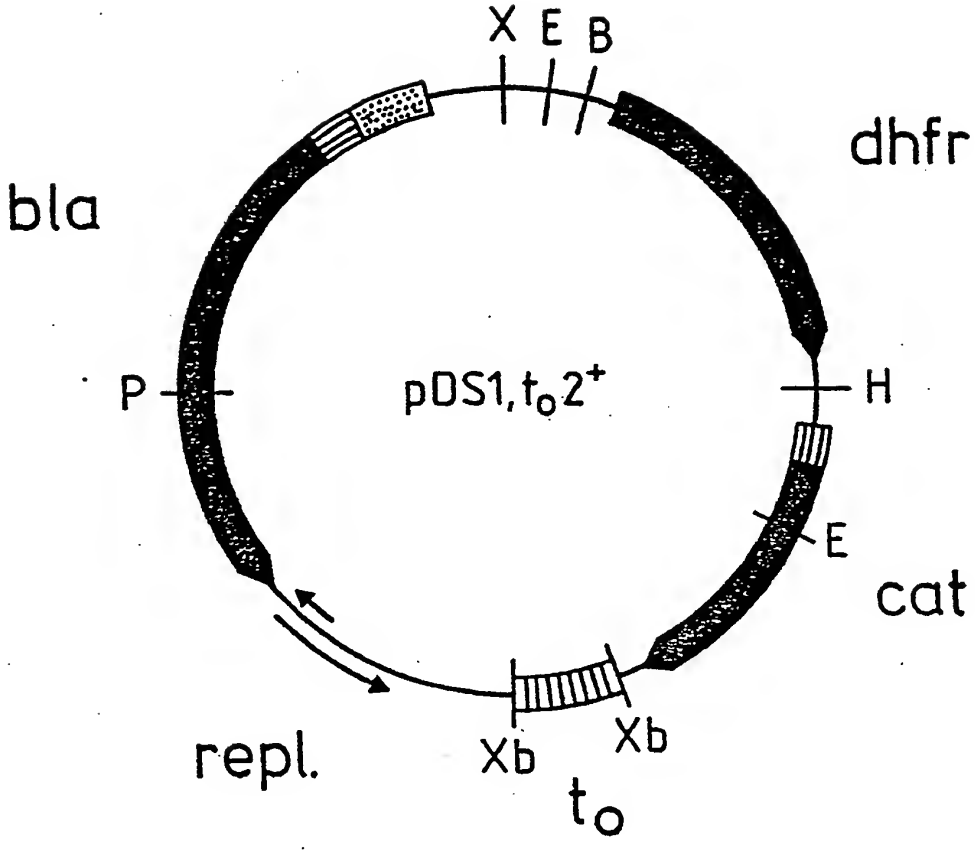

Fig. 4b

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4358

Fig. 5a

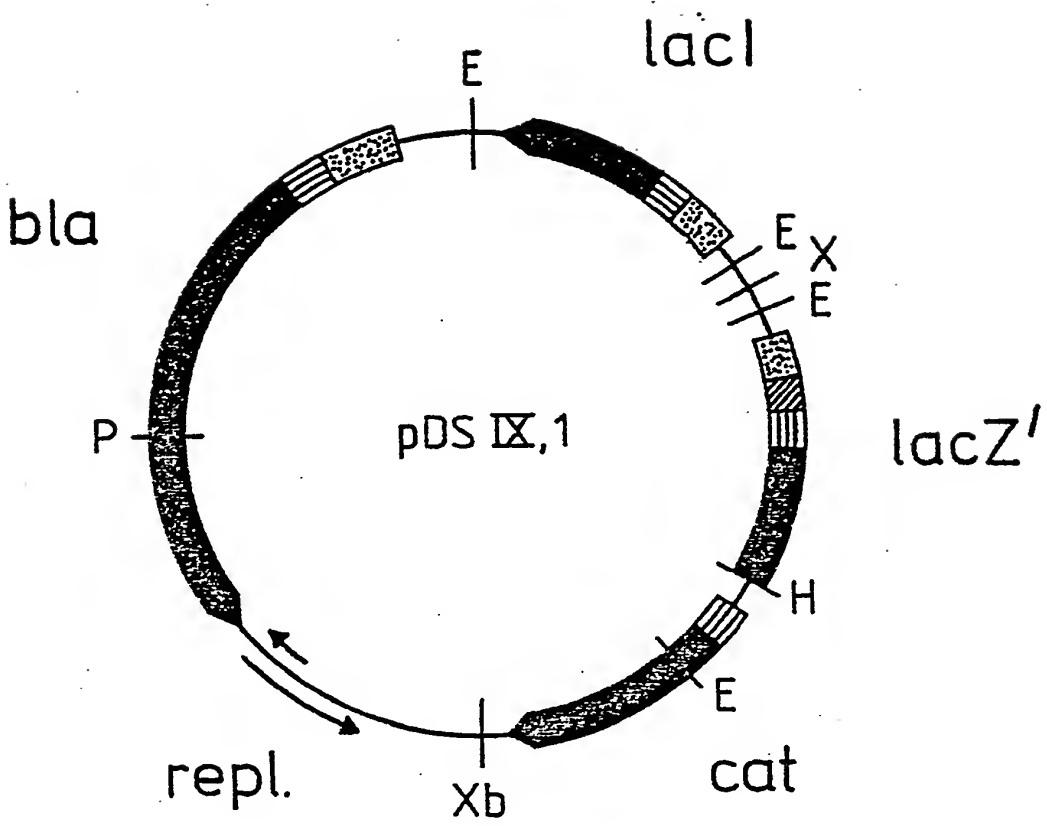

Fig. 5b

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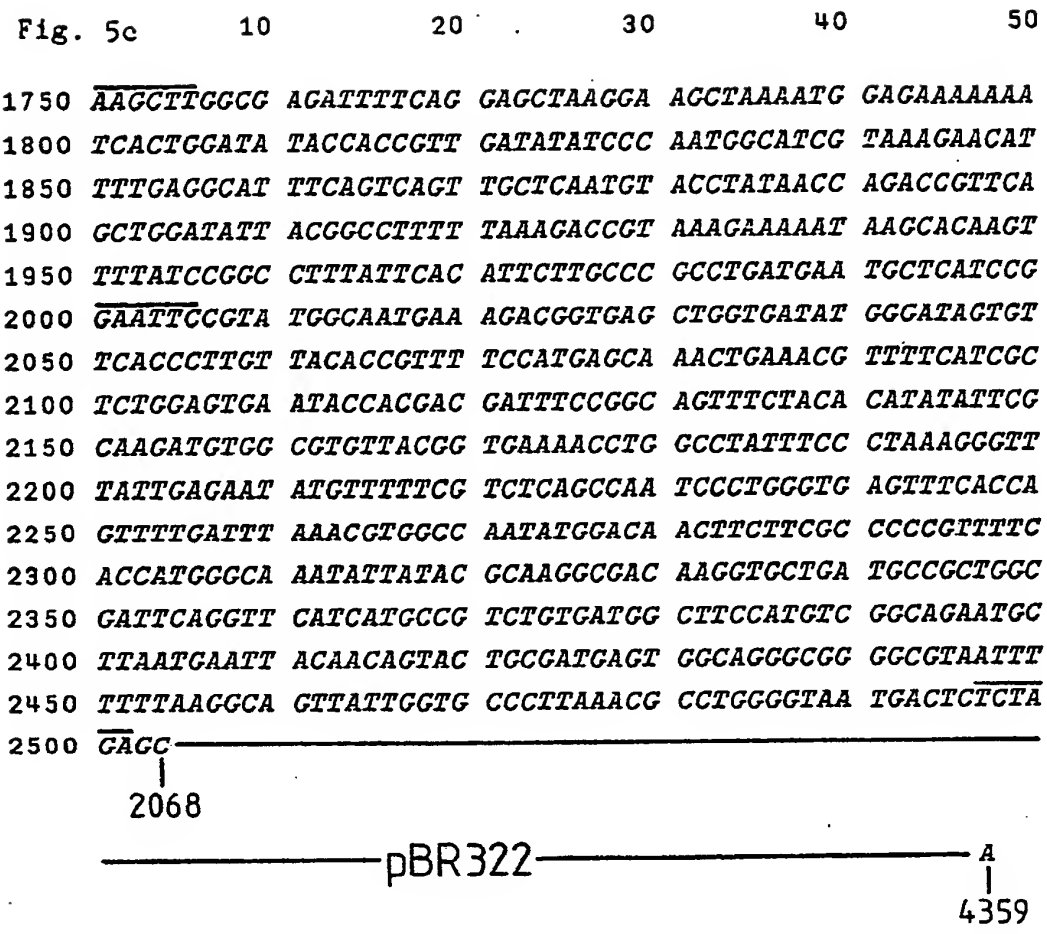
Fig. 5c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30
40
50
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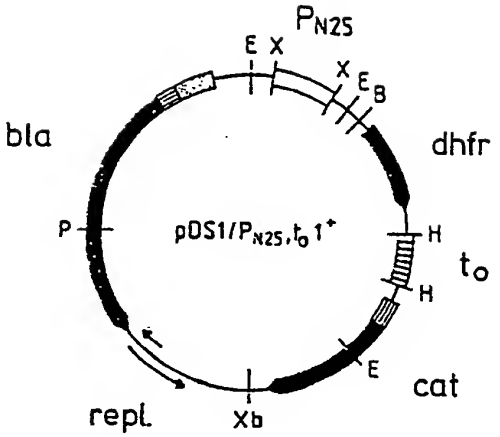
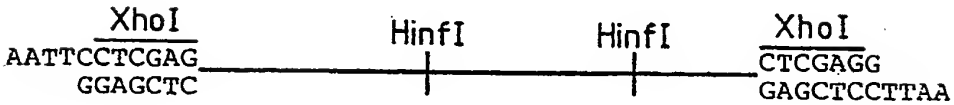
2068
pBR322
A
4359

Fig. 6a

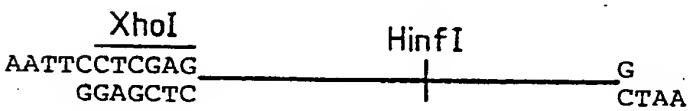


EcoRI

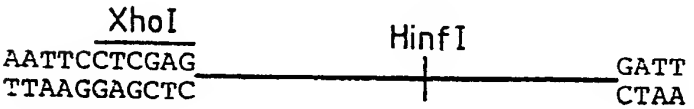
fragment isolation



HinfI, limited digestion



DNA PolI + dNTP's



fragment 1

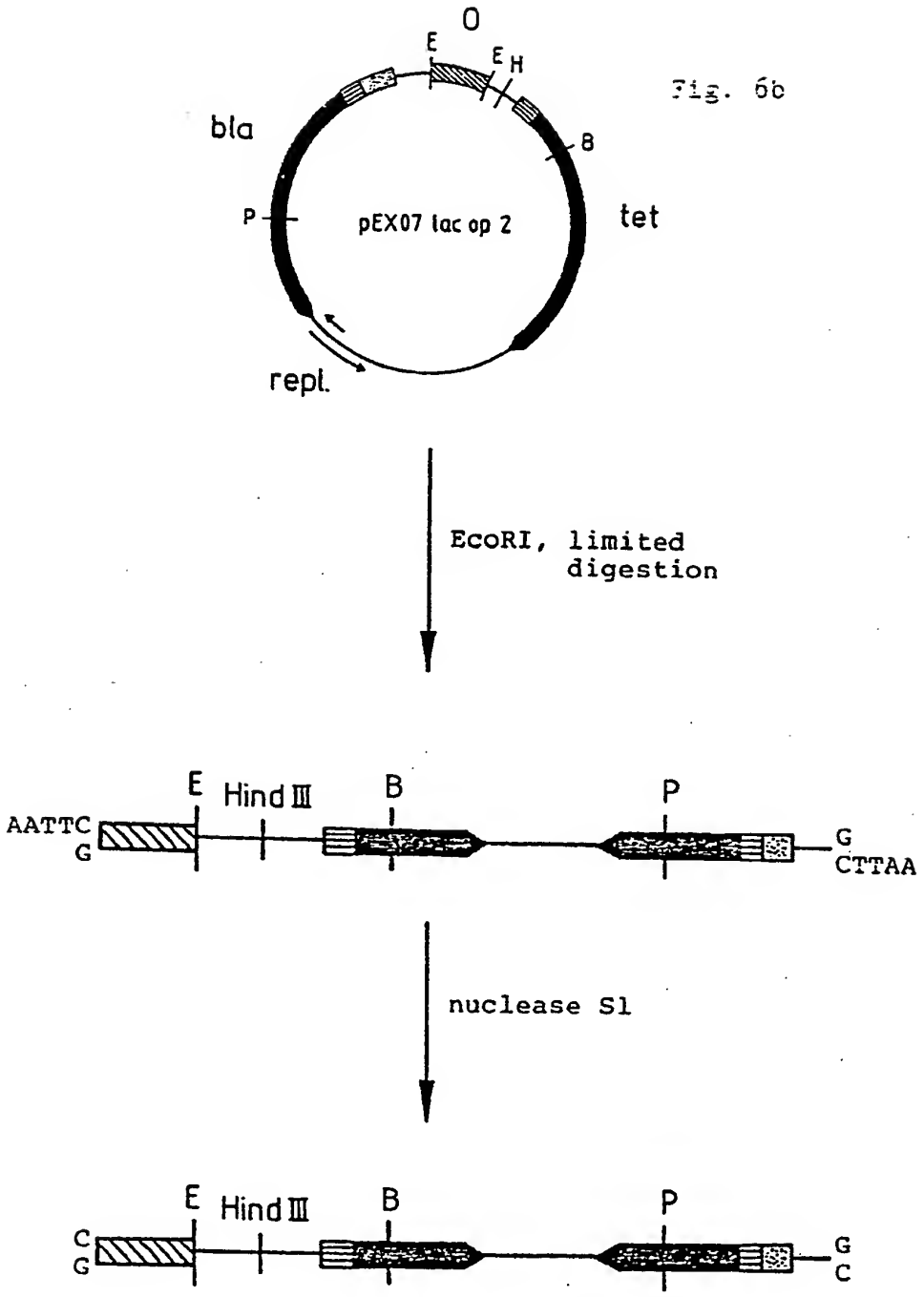


Fig. 6b

fragment 2

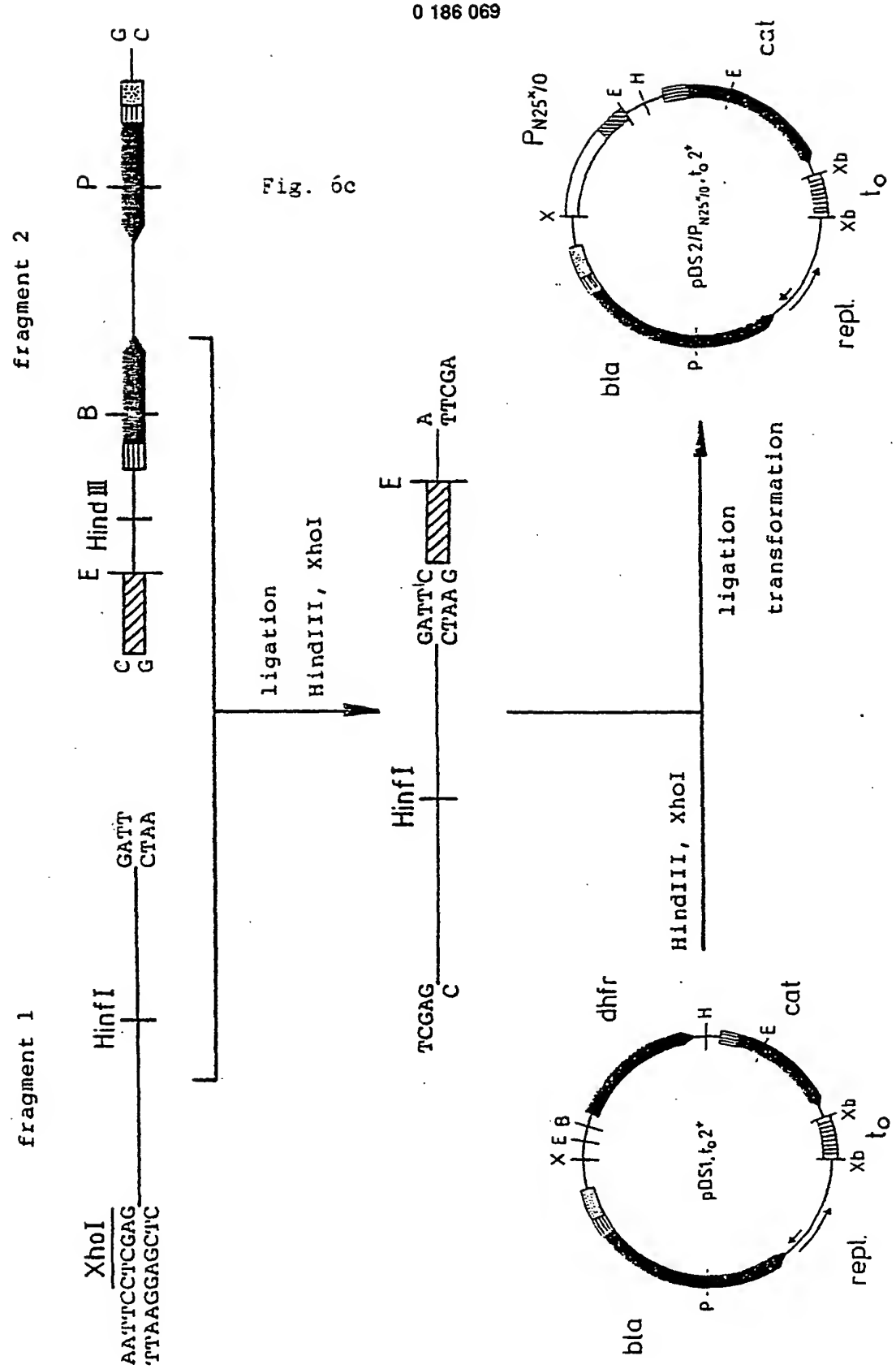

fragment 1
fragment 2
XhoI
HinfI
HindIII
P
B
E
GATT
CTAA
TCGAG
C
GATTC
CTAAG
A
TTCGA
ligation
HindIII, XhoI
transformation
bla
repl.
dhfr
cat
pDS1,t0 2'
pDS2/PN25*10,t0 2'
PN25*10
t0
Xb
H
X E B

Fig. 6c

Fig. 7

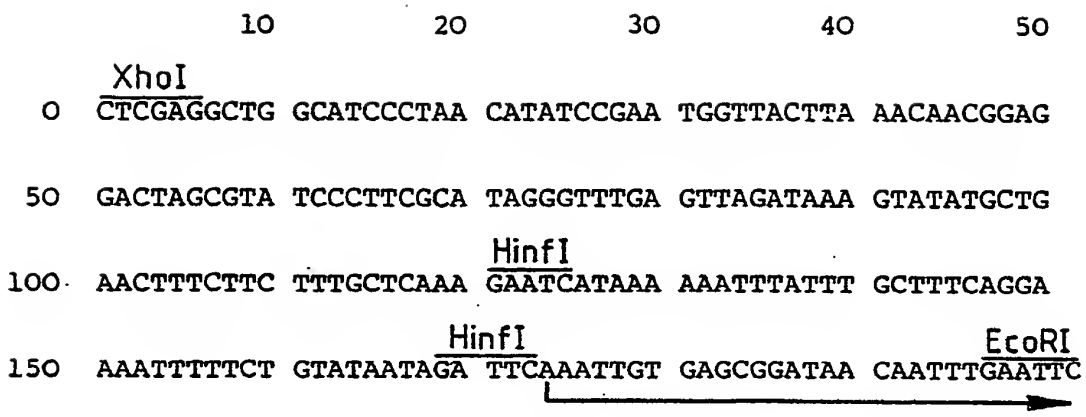
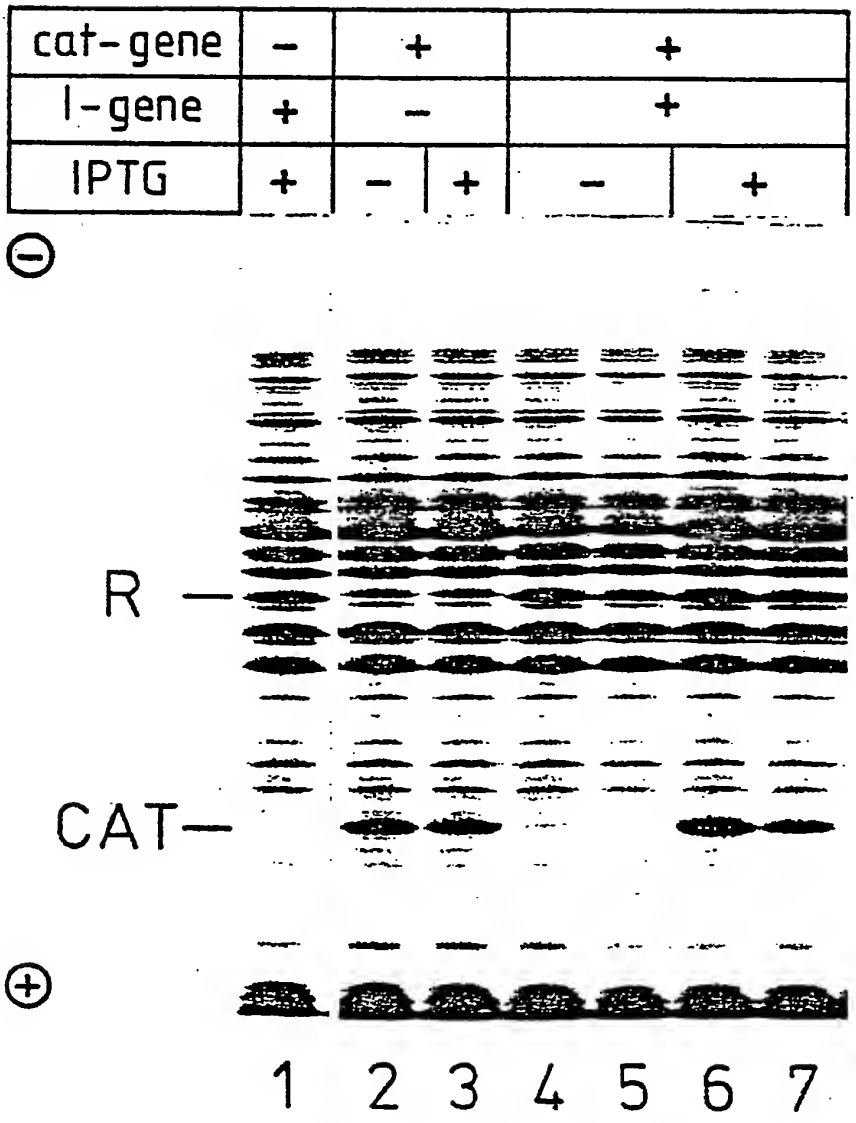

10
20
30
40
50
XhoI
0 CTCGAGGCTG GCATCCCTAA CATATCCGAA TGGTTACTTA AACAACGGAG
50 GACTAGCGTA TCCCTTCGCA TAGGGTTTGA GTTAGATAAA GTATATGCTG
HinfI
100 AACTTTCTTC TTTGCTCAAA GAATCATAAA AAATTTATTT GCTTTCAGGA
HinfI
EcoRI
150 AAATTTTTCT GTATAATAGA TTCAAATTGT GAGCGGATAA CAATTTGAATTC

Fig. 9



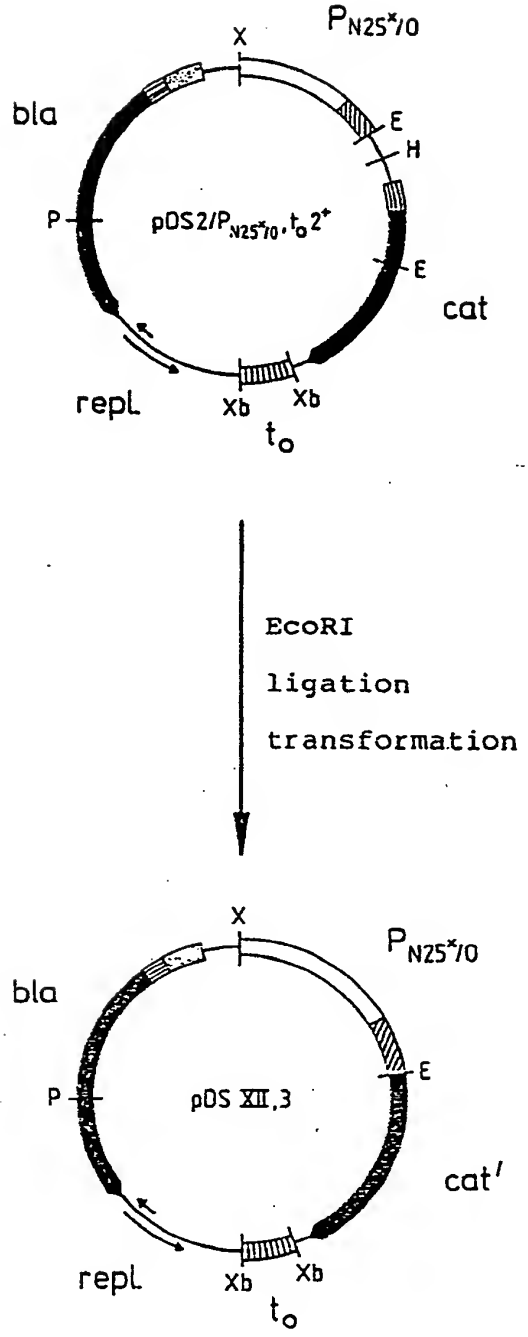

X
$P_{N25^{*}/0}$
bla
E
H
P
pOS2/$P_{N25^{*}/0}$,t_o2^{+}
E
cat
repL
Xb
Xb
t_o
EcoRI
ligation
transformation
X
$P_{N25^{*}/0}$
bla
E
P
pOS XII,3
cat'
repL
Xb
Xb
t_o

Fig. 8a

Fig. 8b

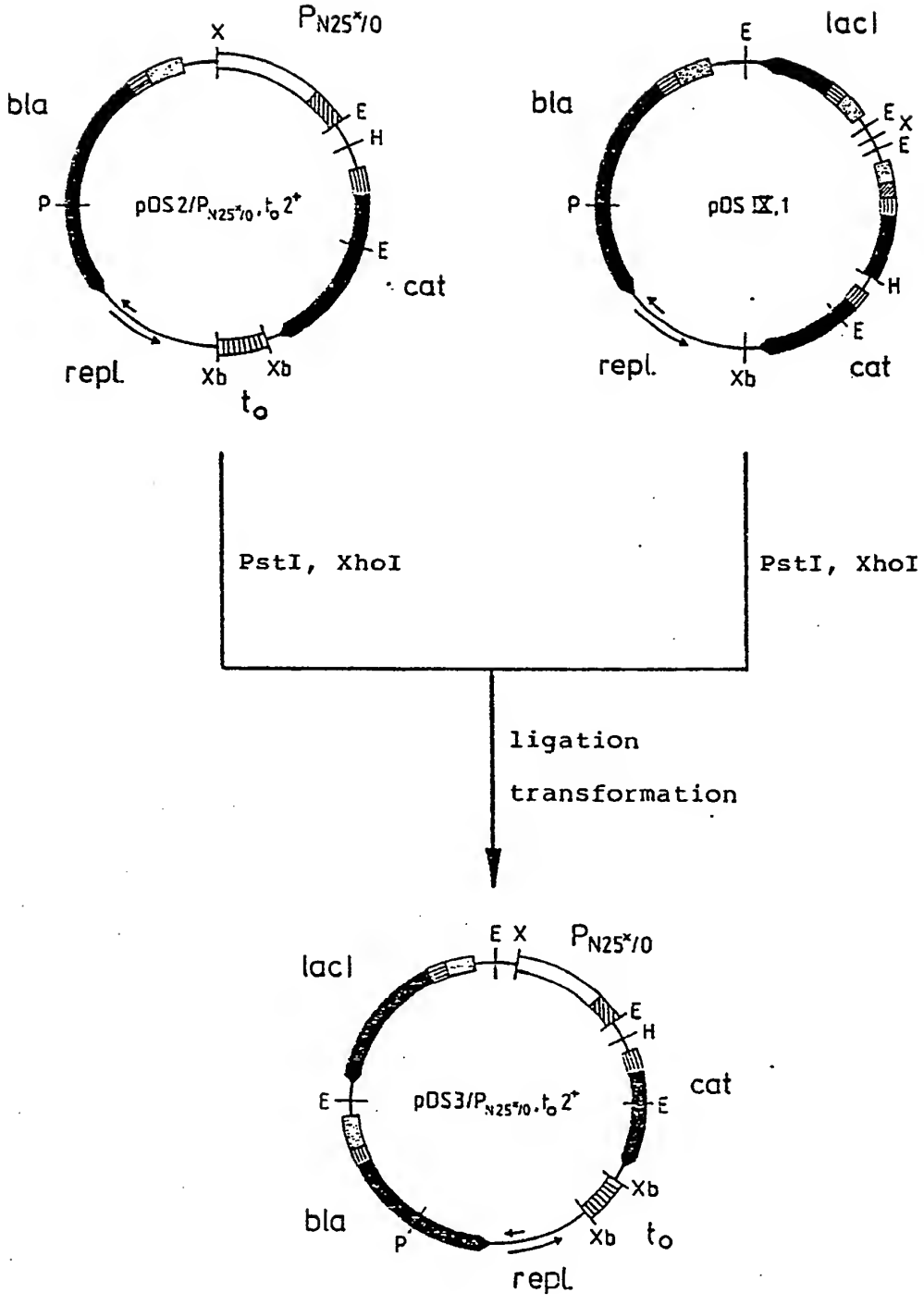
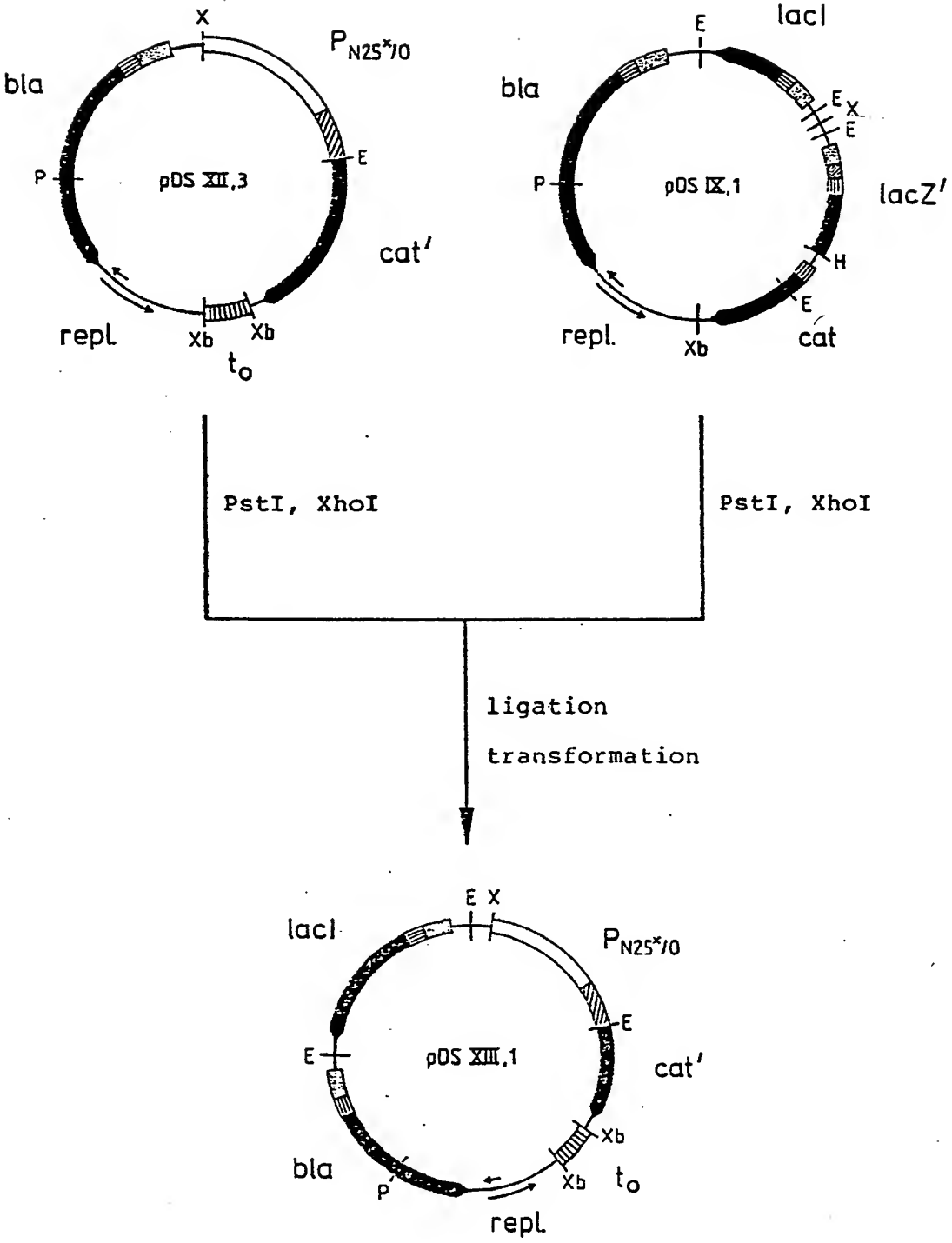

X
$P_{N25^{*}/0}$
bla
E
H
P
pOS 2/$P_{N25^{*}/0}$, t_o 2+
E
cat
repl.
Xb
Xb
t_o
E
lacI
bla
E
X
E
P
pOS IX.1
lacZ'
H
E
repl.
Xb
cat
PstI, XhoI
PstI, XhoI
ligation
transformation
E X
$P_{N25^{*}/0}$
lacI
E
H
E
pOS3/$P_{N25^{*}/0}$, t_o 2+
E
cat
Xb
bla
P
Xb
t_o
repl.

Fig. 8c


X
$P_{N25^{x}/0}$
bla
P
pOS XII,3
E
cat'
repl.
Xb
Xb
t_o
E
lacI
bla
E X
E
P
pOS IX,1
lacZ'
H
E
repl.
Xb
cat
PstI, XhoI
PstI, XhoI
ligation
transformation
E X
lacI
$P_{N25^{x}/0}$
E
E
pOS XIII,1
cat'
Xb
bla
P'
Xb
t_o
repl.